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We have determined that *PML* is a novel growth suppressor that was found to be translocated from chromosome 15 and fused with the retinoic acid receptor-a gene on chromosome 17 (t(15;17) in acute promyelogenous leukemia (APL). *PML* is a member of the newly identified growing family of RING finger domain proteins that includes the breast cancer susceptibility gene *BRCA*1, *ret*, and T18, whose functions ranged from transactivation of viral genes to DNA repair and extinction of homeotic genes. Expression of *PML* has been shown to be altered in breast cancer. In normal breast specimens, less than 3% of the epithelial cells exhibit *PML* staining, but increasing levels of *PML* was detected as the lesions progress from benign dysplasias to carcinomas. These studies suggest that *PML* may play critical role in breast carcinogenesis. We have been studying the function of *PML* as a transcription regulator of the epidermal growth factor receptor (EGFR) gene. Recently, using a p21 promoter-reporter construct, we identified that the p21 gene is transcriptionally activated by *PML*, independent of p53 in a breast cancer cell line. These results suggest that *PML* may be one of the transcription regulators that mediates p21 gene expression.

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Introduction

Breast cancer is one of the most prevalent malignancies in women and accounts for the highest morbidity among women suffering from cancers (1). The oncogenic development of breast cancer is accompanied by genetic alterations of multiple oncogenes, tumor suppressor genes, and other factors. The collaborative effects of these transforming proteins induce alterations in the cellular biochemical, physiological, and genetic processes, which include both gene induction and gene repression, alterations in growth requirement, and acquisition of metastatic potential. These changes may lead to neoplastic transformation of the mammary tissue. The complexity and heterogeneity of the array of genetic, hormonal, and dietary factors that may contribute to the etiology of breast cancer is further confounded by the lack of information on specific genetic mutations associated with the initiation and progression of the disease.

Overexpression of the epidermal growth factor receptor (EGFR), HER-2/*neu*, and *myc* oncogenes are some of the well described genetic changes that frequently occur in breast cancer (2-4). In addition, deletions of chromosomal loci that are thought to be associated with putative tumor suppressors including the p53, BRCA1 and BRCA2 genes, also contribute to a more aggressive phenotype of breast cancer (5). These genetic changes have important prognostic implication in the clinical outcome of breast cancer.

Epidermal growth factor (EGF) is a small polypeptide that stimulates cell proliferation in both cell culture and in intact animals (6). EGF has been shown to promote both normal and neoplastic growth of mammary tissue in rodents (7) and in human breast cancer cells in culture (8-10). The biological effects of EGF are mediated through high affinity binding to EGFR, which is a 170 kDa membrane receptor tyrosine kinase (6). There is great interest in the study of EGFR in human breast cancer, however, the clinical relationships and prognostic value of the receptor in breast cancer are still unclear (11).

The transforming growth factor-α (TGFα), which bears considerable sequence homology to EGF and is produced by many transformed cells, also binds to EGFR and mimics the action of EGF (6,12). Expression of EGFR in breast cancer cells is regulated by mitogenic growth factors, and also by the superfamily of nuclear hormone receptors, which includes estrogen, progesterone, glucocorticoids, and retinoic acid receptors (13). Therefore, optimal regulation of EGFR expression is a complex process involving the coordinate interaction of several heterologous growth factors and hormones, whereby the proliferation of normal and neoplastic breast cells can be modulated. Although the clinical significance and prognostic value of EGFR in human breast cancer are unresolved, the involvement of EGFR in the growth of normal and malignant human mammary cells indicates that it may play a critical role in the oncogenesis of human breast carcinomas. Further evidence of the importance of EGFR in the development of breast cancer has been shown in transgenic mice studies where the overexpression of TGFα causes a significant increase in the occurrence of mammary carcinomas (14-16).

Specific chromosomal abnormalities occur frequently in acute and chronic leukemias (17). These cytogenetic aberrations are thought to contribute to leukemogenesis. Furthermore, significant differences in the type of genes involved in chromosomal translocations in acute leukemias and chronic leukemias have also been observed. For example, study of the recombination of *bcr* and *abl* genes in the t(9;22) of CML, and the *myc* and immunoglobulin genes in the t(8;14) of Burkitt lymphoma, has led to the identification of new fusion genes involved in the neoplastic transformation of these hematopoietic tumors (17-19). It has also been shown that a chromosomal translocation breakpoint t(15;17) occurs in over 90% of all patients with acute promyelocytic leukemia (APL), a subtype of acute myeloblastic leukemia (20-23). The recombination involves the *PML* (*myl*) gene on chromosome 15 and the retinoic acid receptor-α (RARα) on chromosome 17. The chimera *PML*/RARα and RARα/*PML* genes are formed as a result of the reciprocal translocation between the *PML* and RARα loci (20-22, 24). The

PML/RARα cDNA has been isolated and shown to encode a fusion protein that is retinoic acid responsive and exhibits transactivation potential in a cell type- and promoter-specific manner differing from the wild-type RARα (25-27). Since the administration of all-trans retinoic acid to APL patients leads to rapid achievement of remission, it has been suggested that disruption of RARα may be part of the underlying pathogenesis of APL (28-30). The biological function and etiologic implications for the *PML* gene is not known and leaves open the question of its role in APL. Characterization of *PML* reveals that it is a putative zinc finger protein and transcription factor that shares homology with a newly recognized family of proteins that includes a variety of putative transcription factors as well as the recombination-activating gene product (RAG-1) (25-27,31). Expression of *PML* is found in a variety of fetal and adult tissues including brain, gut, liver, lung, muscle, placenta, and testes (31,32).

Our laboratory, in collaboration with Dr. Kun-Sang Chang at the University of Texas, M.D. Anderson Cancer Center, investigated the role of t(15;17) chromosomal translocation in the leukemogenesis and the emergence of multidrug resistance in acute promyelocytic leukemia (APL). We demonstrated that *PML* suppresses the clonogenicity and tumorigenicity of the APL-derived NB4 cells in soft agar (33). Cells transfected with expression vector containing *PML* showed more than 50% reduction in colonies formed on soft agar. Cells transfected with control plasmid (pSG5) and *PML* mutants (PSG5*PML*mut and pSG5*PML*/RARα) did not show inhibition of colony growth. Furthermore, we also show that *PML* suppresses the transformation of REF and NIH3T3 cells by oncogenes. In all of these experiments the fusion product *PML*/RARα fails to suppress the tumorigenic growth of NB4 cells as well as the transformation of the REF and NIH3T3 cells. These results suggest that the translocation of APL inactivated the biological function of *PML* as a tumor suppressor and that this molecular alteration may be a precipitating event in the development of APL.

To assess the putative function of *PML* and *PML*/RARα as a transcription factor, we examined their ability to transactivate promoter activity. Our results showed that *PML* significantly represses the activity of the EGFR gene promoter. However, cotransfection with *PML*/RARα exhibited significantly decreased suppression of the EGFR promoter. These studies showed that *PML* acts to suppress the transcriptional activity of specific gene promoter and that mutant *PML* (*PML*/RARα) lost its transrepression function.

The results of our experiments suggest that the negative regulation of EGFR expression by *PML* may attenuate growth of mammary tissue normally, while genetic alterations of PML may lead to the neoplastic growth of the tissue. Our laboratory, therefore is interested in exploring the role of *PML*, a novel growth suppressor, and its regulation of EGFR expression in the genetic and molecular etiology of breast cancer.

Body

In this grant period (August 1, 1995 - July 31, 1996), we have attempted to transfect the EGFR promoter-reporter construct in a variety of breast cancer cell lines including SKBr3, T47D, MCF-7, MDA-MB-231, MDA-MB-435, MDA-MB-468, and MDA-MB-453, but have not successfully obtained reasonable reporter expression; and therefore, unable to verify the transactivation or repression of the EGFR promoter by *PML*. The inability to express the EGFR promoter-reporter construct in these breast cell lines suggest that the 1.1 kb promoter may lack the critical regulatory or tissue-specific enhancer sequence that confers optimal expression in breast cells. We then isolated and obtained a 2.2 kb EGFR promoter fragment from a genomic library, containing in addition, approximately 1 kb of nucleotide sequence further upstream from the original promoter construct that we have. We cloned this 2.2 kb promoter fragment into a CAT vector and then attempted to transfect this reporter construct into the breast cancer cells as described above, but again failed to obtain reasonable CAT expression, and observed neither transactivation nor repression by *PML*.

In contrast to the human adrenocortical carcinoma SW13 cells, which we have previously successfully used as a model system to study the expression of the EGFR promoter-reporter and its negative regulation by *PML*, it is unclear why these breast cancer cells did not confer expression for the EGFR promoter. The transfection efficiency may not be a problem, because we have used these cells to study the expression of other promoter-reporter constructs and found satisfactory expression of the reporter gene. However, it is conceivable that the EGFR promoter construct that we have contained repressor elements, which prevent the expression of the reporter gene. We then transfected various deletion mutants of the promoter into SKBr3 cells, one of the breast cancer lines, and again unable to detect the expression of the reporter driven by the EGFR promoter nor regulation by *PML*.

Current work in the lab focuses on characterization of *PML* expression by immuno-histochemistry in paraffin embedded section derived from patients' breast cancer samples. We are also carrying out Southern and Northern blotting to assess the status of *PML* gene in various human breast cancer cell lines. Patients cancer samples are also being obtained from the Tissue Retrieval Service of The Cancer Institute of New Jersey for these analyses.

More recently, using a p21 promoter-reporter construct, we identified that the p21 gene is transcriptionally activated by *PML*, independent of p53 in the breast cancer cell line SKBr3. 1 gene is transcriptionally activated by *PML* in a breast cancer cell line, SKBr3 (Fig. 1). The wild-type p53 control conferred approximately 7-fold activation of the promoter, whereas a p53 mutant did not transactivate the promoter. The induction of the p21 promoter activity by *PML* is about 4 to 5-fold. However, the fusion mutant *PML*/RARα induced a 20-fold increase in the activity of the promoter. These results suggest that *PML* may be one of the transcription regulators that mediates p21 gene expression. Addition of retinoic acid did not alter the induction. In addition, introduction of the retinoic acid receptor-α (RARα) in the presence or absence of retinoic acid did not produce an induction, suggesting that *PML* is a transcription activator for p21 and when it is fused with RARα, the transactivation potential of *PML* is increased. Since the SKBr3 cells express mutant p53 protein, we decided to verify these findings in the $p53^{-/-}$ human lung carcinoma H1299 cells (34). We observed that both *PML* and the mutant *PML*/RARα consistently stimulated the p21 promoter activity by about 5-fold (Fig. 2). Surprisingly, in the presence of retinoic acid, the induction in mutant *PML*/RARα was further increased. The RARα transfected cells did not show significant induction of the p21 promoter either in the presence or absence of retinoic acid. These results further suggest that *PML*, like p53, is a transcription activator of the p21 gene and may play key role in breast oncogenesis.

Conclusions

Through a series of exhaustive studies, we were unable to obtain expression from the EGFR promoter-reporter constructs nor observed regulation imposed by *PML*. It is conceivable that the EGFR promoter that we obtained lacks critical regulatory or tissue-specific enhancer sequence that permit expression in breast cells.

Ongoing efforts are currently directed at studying the genetic status of *PML* gene in breast cancer samples by DNA and RNA hybridization, and also immunohistochemistry with paraffin embedded samples.

We have recently determined that the cell cycle regulator p21 is also a target for transactivation by *PML*. Genetic alterations of *PML* including gene mutations, and aberrant chromosomal translocation such as those in acute promyelogenous leukemia involving RARα, may be common in breast cancer. Indeed expression of *PML* has been shown to be altered in breast cancer (35). In normal breast specimens, less than 3% of the epithelial cells exhibit *PML* staining, but increasing levels of *PML* was detected as the lesions progress from benign dysplasias to carcinomas. *PML* expression is also markedly reduced when malignant cells become invasive

(35). These studies further suggest that *PML* may play a critical role in breast carcinogenesis. We reasoned that aberrant regulation of p21 by *PML* may contribute to the development of breast cancer. Future studies will involve the characterization of the regulatory relationship between *PML* and p21.

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Appendix

Figure 1

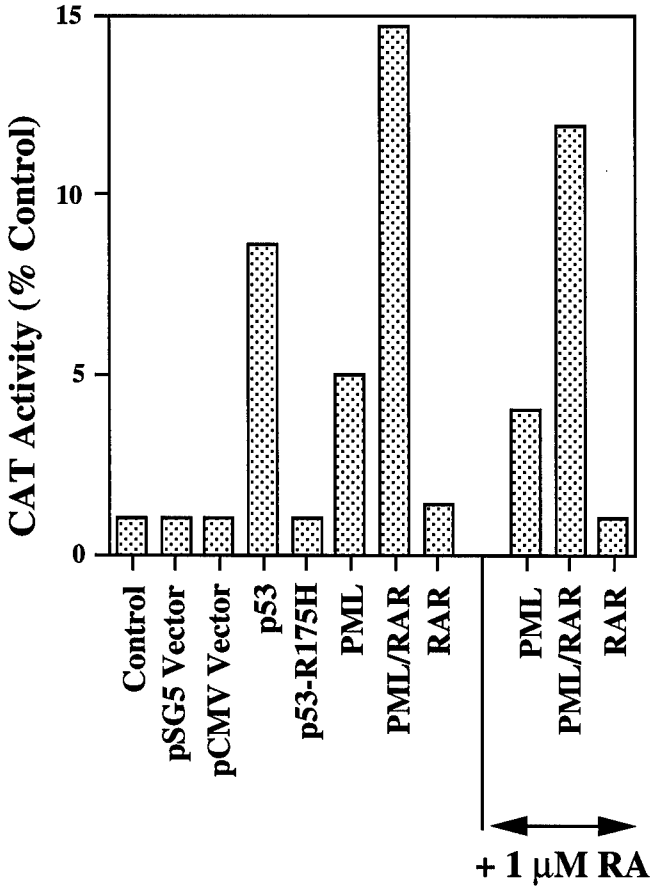

Figure 2